Genetic Strategies to Enable Sustained Expansion and Immortalization of Human Erythroid Progenitors for On-Demand Blood Production

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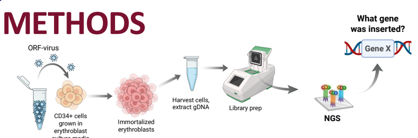
BACKGROUND

- Reliable access to red blood cells (RBCs) is critical for military medicine, particularly in austere or remote environments where blood supply is increasingly challenging.
- One promising solution is **the development of *ex vivo* expandable erythroid progenitor systems capable of generating RBCs on demand.** However, limited proliferative capacity and rapid erythroid progenitor differentiation remain major barriers to scalable RBC production.
- Here, we describe the strategy for **identifying factors capable of expanding erythroid progenitors.**

SPECIFIC AIMS

- Identifying candidate genes through an unbiased open reading frame (ORF) library, screening for genes involved in slowing the differentiation and increasing the self-renewal ability of human erythroblasts from CD34⁺ hematopoietic stem cells.
- Systematic evaluation of a curated list of 7 genetic factors previously shown to increase expansion or immortalization of erythroblast progenitor cells *ex vivo.*

METHODS



- We performed a pooled gain-of-function screen using an ORF library representing >16,000 human genes. Human CD34⁺ hematopoietic progenitor cells were cultured under erythroid conditions and transduced with the ORF library. Cells were maintained for up to 32 days to assess morphology, survival, and clonal dynamics.
- Enriched ORFs were identified by next-generation sequencing (NGS). In parallel, 7 candidate genetic factors (Table 1) were evaluated for effects on erythroid progenitor expansion and differentiation.

RESULTS: ORF Library Screen

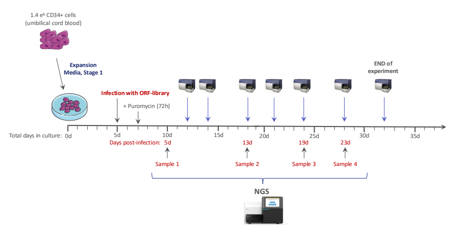


Figure 1. Graphical summary of the ORF library screening approach for identifying genes involved in expansion and self-renewal of human erythroblasts. Human CD34⁺ hematopoietic progenitor cells were cultured under erythroid conditions and transduced with the ORF library. The culture was monitored for up to 32 days to assess clonal expansion, progenitor surface marker expression, and morphological characteristics. Cell pellets were collected at multiple time points (days 5, 10, 13, 19, and 23) for genomic DNA isolation and next-generation sequencing (NGS) to track ORF representation.

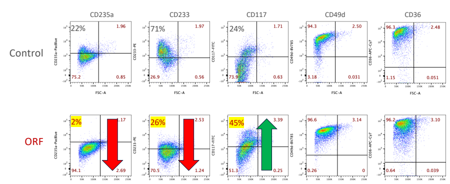


Figure 2. ORF-transduced cells retained a progenitor-like surface marker profile relative to controls. At day 12 post-transduction, ORF-expressing cells exhibited substantially higher expression (45%) of the early hematopoietic progenitor marker c-KIT (CD117), accompanied by reduced expression of mature erythroid markers CD235a (2%) and CD233 (26%) compared to control. Expression of progenitor and erythroid markers analyzed by flow cytometry, 12 days post ORF-transduction. Control cells represent untransduced CD34⁺ progenitor cells grown under same conditions as transduced cells.

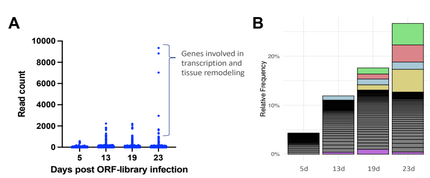


Figure 3. Next generation sequencing analysis revealed selective enrichment of specific ORFs/genes involved in transcription and tissue remodeling over time, with the strongest enrichment observed at day 23 post-transduction. A. ORF enrichment across samples. The Y-axis represents normalized ORF read counts, with each blue dot representing individual ORFs. **B.** Top 50 ORF read frequencies across samples. ORFs present in >1% of samples are highlighted.

RESULTS: 7-Factor Screen

Factors	Function	Reference
JAK2V617F	Constitutively active JAK2 kinase	Levine, R.L. *et al.* (2005)
GATA1	Megakaryocyte-Erythroid maturation transcription factor	Stachura, D. L., Chou, S. T. & Weiss, M. J., (2006)
AML-ETO	Transcriptional regulation, fusion protein	Tonks, A. *et al.* (2003)
BMI1	Cell cycle, self-renewal regulator	Liu, S. *et al.* (2021)
HPV16-E6/E7	p53/pRB suppressor	Kurita, R. *et al.* (2013)
MYC	Transcription factor	Delgado, D & Leon, J., (2010)
BCL-X_L	Anti-apoptotic protein, that prevents release of cytochrome c from mitochondria	Hirose, S. *et al.*, (2013), Bird *et al.*, (2014)

Table 1. Curated list of oncogenic factors that have previously been reported to be involved in immortalization and/or the proliferation and maintenance of erythroblast populations.

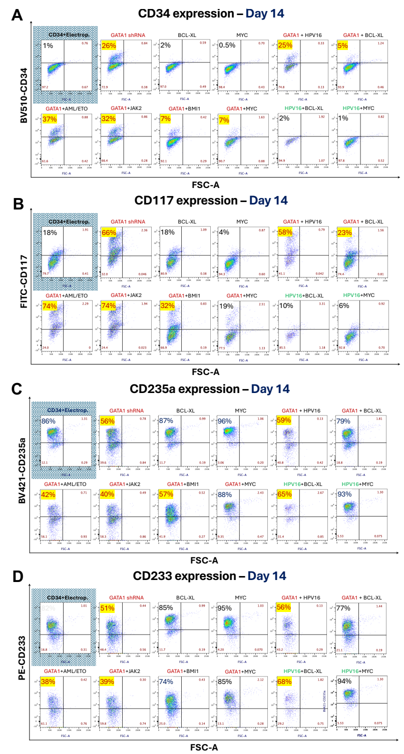


Figure 4. Nucleofection with GATA1 shRNA or an HPV16-E6/E7 overexpression vector resulted in a pronounced and sustained enrichment of immature erythroid progenitor markers relative to control cells. Expression of progenitor (**A, B**) and erythroid (**C, D**) markers analyzed by flow cytometry, 14 days post nucleofection. Control cells represent electroporated CD34⁺ hematopoietic progenitor cells grown under same conditions as nucleofected cells.

CONCLUSIONS

ORF Library Screen

- ORF-transduced cells demonstrated sustained expression of early progenitor markers, reduced expression of mature erythroid markers, enhanced clonal expansion capacity compared to untransduced controls
- Identification of these regulators provides a foundation for engineering erythroid progenitor systems capable of sustained expansion and conditional immortalization, supporting development of scalable RBC production platforms for military and civilian transfusion applications.

7-Factor Screen

- Among the factors tested, nucleofection with **GATA1 shRNA** or an **HPV16-E6/E7 overexpression vector** resulted in a pronounced and sustained enrichment of immature erythroid progenitor markers relative to control cells.
- Our findings demonstrate that enforced expression of specific genes can delay erythroid differentiation and enhance persistence of human erythroid progenitors during *ex vivo* culture.

FUTURE WORK

- Evaluate which factors, alone or in combination, promote immortalization of erythroblasts or increase expansion/slow down differentiation of erythroid progenitors.
- Develop inducible expression systems to evaluate whether transient expression can support reversible erythroid progenitor expansion while preserving differentiation potential.

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